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Evidence for reversible multiple redox transformations of [3Fe-4S] clusters

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Analysis of the diffusionless cyclic voltammetry of ferredoxin III from *Desulfovibrio africanus*, co-adsorbed with neomycin as an electroactive film on pyrolytic graphite 'edge' electrodes, shows that the reduced $[3Fe\text{-}4S]^{(0)}$ cluster undergoes further fast, chemically reversible, *two*-electron reduction, at a potential $E^{0\prime}$ = ca. −720 mV (pH 7.15, 0°C). The pH dependence of $E^{0\prime}$ (pH 6.25–7.80) indicates net transfer of *two* H^+. Observation of similar voltammetric waves in other proteins specifically containing [3Fe-4S] centres suggests that extensive redox activity and stabilisation of what is formally an all-Fe(II) species, may be a common, perhaps characteristic, feature of this cluster type.

Ferredoxin; Iron-sulphur; Electron transfer; Voltammetry; Electrochemistry; (*Desulfovibrio africanus*)

1. INTRODUCTION

In recent publications, we have described electrochemical and spectroscopic investigations of two ferredoxins, Fd I from *Azotobacter chroococcum* [1] and Fd III from *Desulfovibrio africanus* [2]. Each of these contain two clusters, one [3Fe-4S] and one [4Fe-4S]. Interaction of the proteins at pyrolytic graphite 'edge' (PGE) electrodes is promoted by certain aminoglycosides, such as neomycin, that contain an array of several $\text{-}NH_3^+$ groups. To our surprise, cyclic voltammetry thus obtained on solutions of these ferredoxins has revealed *three* redox couples. Two were assigned in each case to the $[3Fe\text{-}4S]^{1+/0}$ and $[4Fe\text{-}4S]^{2+/1+}$ systems while the additional feature at lower potential, was not characterised further. However, in the case of Fd III which contains only seven cysteines (all of which are implicated in cluster binding) the reasonable deduction is that this couple corresponds to further reduction of one of the clusters. Moreover, its disappearance *in concert* with the transformation of $[3Fe\text{-}4S]^0$ into $[4Fe\text{-}4S]^{2+}$ (a reaction of Fd III that occurs rapidly in the presence of Fe^{2+} [3]) suggests, specifically, an association with the [3Fe-4S] cluster.

It thus follows that [3Fe-4S] clusters may be capable of extensive reversible redox activity that has previously remained undetected. Such a property would represent a significant departure from the now-established idea that reactions of biological Fe-S clusters are tuned tightly by the protein environment to span just two adjacent oxidation levels [4]. (Contrast the *extensive sequential* redox transformations of synthetic analogues $[Fe_2S_2(S_2R)_2]^{n-}$ and $[Fe_4S_4(SR)_4]^{n-}$ [5].)

To analyse this observation in more detail we have exploited the finding that Fd III adsorbs strongly at PGE in the presence of neomycin. This was evident initially from the solution voltammetry, through measurements of peak currents and the appearance of waveforms as the scan rate was varied [2]. Voltammetry of thin films, and particularly of strongly adsorbed molecular arrays, affords a direct means to characterise proteins with complex multiple redox activity. By contrast with the voltammetry of freely diffusing species in solution, the waves appear as signals that have an essentially finite width and their integrated intensities yield absolute values for the number of electron equivalents exchanged with the electrode [6]. For systems featuring several redox couples that do not overlap in potential, relative electron stoichiometries may thus be established directly.

2. MATERIALS AND METHODS

Ferredoxin III was isolated from *Desulfovibrio africanus*, strain Benghazi (NCIB 8401) using the procedure described previously [7]. Protein solutions having a purity index (A_{408}/A_{280}) of 0.78 were stored as pellets in liquid nitrogen. Concentrations were determined using an extinction coefficient 28.6 $mM^{-1}\cdot cm^{-1}$ at 408 nm. Solutions for electrochemistry were prepared by diafiltration (Amicon 8MC, YM5 membrane) into the electrolyte solution. This contained 0.10 M NaCl (BDH Aristar) with a mixture (5 mM in each) of Taps, Hepes, Mes (all Sigma) and acetic acid (BDH). To this were added aliquots of concentrated stock solutions of neomycin (Sigma) and EGTA (BDH) to give final concentrations of 2 mM and 0.10 mM, respectively. At appropriate stages, the pH was measured using a

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microcombination pH electrode (MI-410, Microelectrodes) and adjusted by additions of NaOH or HCl solutions.

The glass cell for cyclic voltammetry and preparation of PGE electrodes have been described previously [8]. The SCE reference electrode was thermostatted at 25°C (E_{SCE} = +244 mV vs SHE) while the sample compartment was maintained at 0°C by immersion in melting ice or a cooling bath. Cyclic voltammetry was carried out using an Ursar Instruments potentiostat and voltammograms were recorded on a Bryans Gould 60000 XY recorder. Measurements were made typically following 10 cycles at 500 $mV \cdot s^{-1}$. All potentials given have been corrected to correspond to the SHE.

Adsorbed films of Fd III were prepared by spreading a ~1 µl volume of ice-cold protein solution onto the surface (area ~0.2 cm^2) of a freshly polished chilled PGE electrode. The protein was typically 100 µM in electrolyte containing neomycin and adjusted to pH 7.0. After removing the excess solution with a tissue the electrode was transferred to the voltammetry cell where it was immersed in the desired Ar-purged electrolyte solution.

3. RESULTS AND DISCUSSION

As shown in fig.1 (upper) and fig.2, films of Fd III prepared as described give cyclic voltammograms that show 3 pairs of waves. We have termed these A′, B′ and C′; their positions correspond closely to those observed (A, B and C) in the solution voltammetry of the protein [2]. In the latter study we assigned A and B to the couples $[3Fe\text{-}4S]^{1+/0}$ and $[4Fe\text{-}4S]^{2+/1+}$, respectively. For comparison, values of $E^{0\prime}$ (= $\{E_{pa} + E_{pc}\}/2$, error ± 10 mV) are: for the film; A′, −140 mV; B′, −390 mV; C′, −720 mV (at pH 7.15, 0°C); for the solution; A, −140 mV; B, −410 mV; C, −726 mV (at pH 7.4, 2°C). For A′(A) and B′(B), $E^{0\prime}$ is essentially invariant with pH. By contrast, couple C′(C) shows pH-dependent behaviour both in terms of peak positions and the shape of the reduction wave. This is clear from inspection of fig.2. Upon increasing the electrolyte pH, reduction wave C′ is noticeably broadened – a feature which is particularly apparent at highest scan rates. For each of the other waves, including oxidation wave C′, peak current amplitudes were proportional to scan rate as is typical of a reversible electrode reaction with adsorbed species [6]. Provided neomycin was present in the electrolyte, films were sufficiently stable, in the pH range 6.25–7.8, to study over time courses of several minutes. At higher pH, it was difficult to obtain adequate coverage, while voltammetry at lower pH showed time-dependent changes in peak positions. Without neomycin in the cell electrolyte, Fd III desorbed rapidly, thus showing that reversible coadsorption of the aminoglycoside was essential for stabilising interactions between Fd III and the PGE surface.

Fig.1 (lower) shows that if the film is scanned in electrolyte containing Fe^{2+}, reductive passage through wave A′ initiates a very rapid transformation in which waves C′ and A′ vanish while B′ becomes larger. This is analogous to the result obtained in solution studies [3] where, in the presence of Fe^{2+}, waves A and C each disappear as $[3Fe\text{-}4S]^0$ is transformed into a second

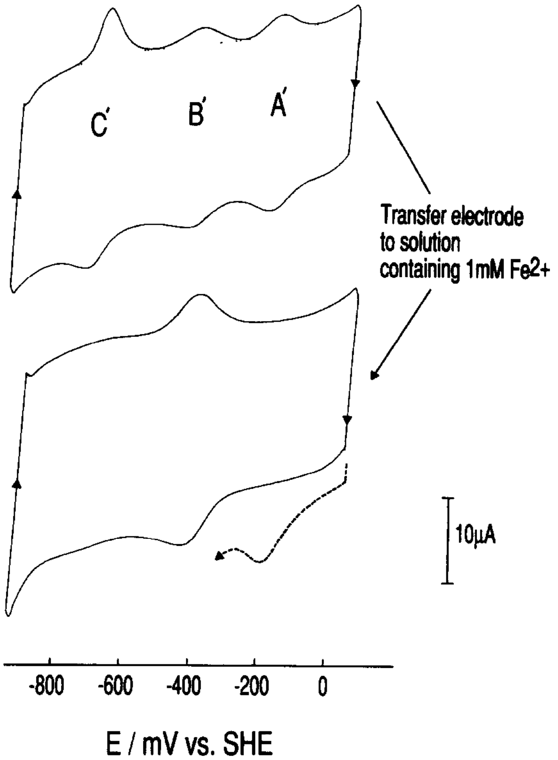

Fig.1. (Upper) Cyclic voltammogram (tenth cycle) of Fd III adsorbed on a PGE electrode in the presence of 2 mM neomycin. Electrolyte pH 6.25; scan rate 500 $mV \cdot s^{-1}$; filter 300 Hz; temperature 0°C. A background trace, typical of those used to determine the charge Q transferred in each electrode reaction, is indicated (···). (Lower) Cyclic voltammogram (fifth cycle) of adsorbed Fd III after transfer to a solution containing 1 mM Fe^{2+}. Passage through wave A′ on the first scan, which is known from solution studies to initiate the [3Fe-4S] to [4Fe-4S] transformation, is shown (···). Electrolyte pH 6.25; scan rate 500 $mV \cdot s^{-1}$; filter 300 Hz; temperature 0°C.

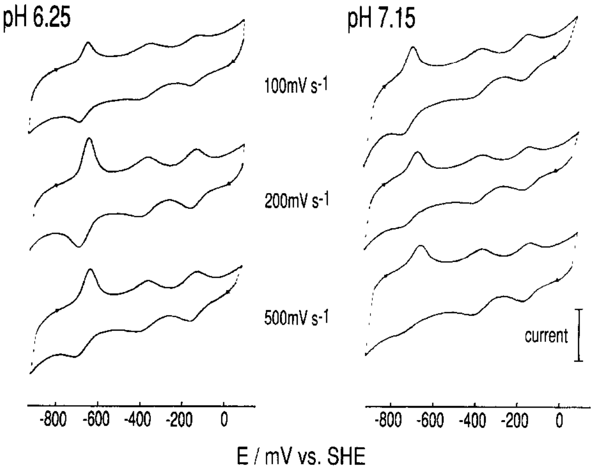

Fig.2. Cyclic voltammograms of adsorbed Fd III, under various conditions of electrolyte pH and scan rate. Temperature 0°C. Capacitance contribution has been trimmed in each case to save space. Each voltammogram was measured on a different film, thus coverages vary. Current scales were: for scan rate 100 $mV \cdot s^{-1}$, 0.5 µA; for 200 $mV \cdot s^{-1}$, 1.0 µA; for 500 $mV \cdot s^{-1}$, 2.0 µA.

[4Fe-4S] cluster. Thus the reactivity that is characteristic of this [3Fe-4S] cluster is *mirrored* in the behaviour of the film. Taking this observation together with the similarity in $E^{0\prime}$ values, we may conclude that molecules of Fd III 'trapped' as an adsorbed array with neomycin, display behaviour that is essentially 'native'. This supports and justifies the quantitative description of the nature of couple C′ that now follows.

The adsorbed film voltammograms show waves C′ as the most prominent feature. This is in contrast to the *solution* voltammetry of Fd III, for which waves C appear as relatively small features whose amplitudes relative to waves A and B vary somewhat erratically (reflecting varied contributions from adsorbed and freely diffusing molecules). Since oxidation C′ waves were always observed to be sharp, it was possible to examine the electron stoichiometry of coupled C′ by determining the charge Q^{ox} *returned* to the electrode from reduced species C′ and comparing this with the charges returned, during the same scan, from A′ or B′. This was achieved by integrating each oxidative wave on the voltammogram using the method of weighing traced cut-outs. Baselines were constructed by aligning the capacitance backgrounds (see, for example, fig.1 (upper)) obtained under identical conditions of electrolyte and scan rate, such that the two made contact tangentially at points approximately midway between peaks. Since the wave envelope for electrode reactions of adsorbed species is compact, even for quite slow electron transfer [6,9], and with such adequate separation of the waves as observed, this procedure was quite appropriate. A precaution implemented was to ensure that the low-limit switching potential was sufficiently low to compensate for kinetic restrictions (slowness of reduction) apparent from the broadening of reduction wave C′. Data obtained by this method were treated by computing the ratios $Q^{ox}(C')/Q^{ox}(A')$ and $Q^{ox}(B')/Q^{ox}(A')$. The latter varied but was always greater than 1.0, as expected in view of the tendency of $[3Fe\text{-}4S]^0$ to convert spontaneously to $[4Fe\text{-}4S]^{2+}$ in the presence of Fe^{2+}. (Even with EGTA present, we have not been able to quench, completely, this reactivity which probably stems from slow decomposition of Fd III and release of Fe.)

As clear from inspection of fig.3, the ratio $Q^{ox}(C')/Q^{ox}(A')$ lies close to 2.0, irrespective of scan rate or pH. The invariance with scan rate makes it most unlikely that C′ is a minority species whose activity is amplified by a coupled chemical reaction. Couple C′ persisted even after holding the electrode at −850 mV for a period of one minute, although analysis at this and longer times was complicated by slow desorption of protein. Individual values of $Q^{ox}(C')/Q^{ox}(A')$ were independent of the corresponding $Q^{ox}(B')/Q^{ox}(A')$ ratio, thereby showing that there is no *direct* relationship between B and C, as indeed expected from the result shown in fig.1 (lower). The earlier case for linking couples C′ and A′ together is now reinforced quantitatively. Since A′, assigned to solution couple A ($[3Fe\text{-}4S]^{1+/0}$), is a *one*-electron process, it follows that C′ must involve *two* electrons. This interpretation is supported, qualitatively, by the shape of reduction wave C′. Widths at half-peak height are typically *less* than the theoretical minimum value of 83 mV (0°) expected for a reversible one-electron process [6] and this indicates a degree of concertedness in the re-oxidation process in which the second electron is somewhat easier to remove than the first. Since Fd III contains no other appropriate redox-active groups, we conclude that couple C′ represents further *two*-electron reduction at the [3Fe-4S] centre. In other words, the voltammetry reveals the sequence described broadly by eqn 1.

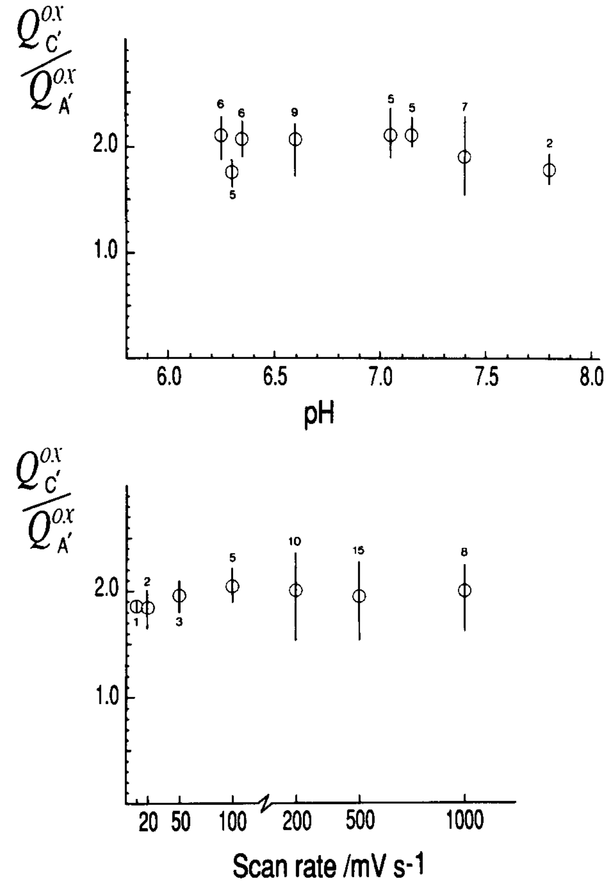

Fig.3. Variation of the ratio $Q^{ox}(C')/Q^{ox}(A')$ with: (upper graph) pH of electrolyte; (lower graph) cyclic voltammetric scan rate. The number of independent determinations made in each case is indicated.

$$\underbrace{[3Fe\text{-}4S]^{1+} \longleftrightarrow [3Fe\text{-}4S]^{0}}_{\text{Couple A}'} \longleftrightarrow \underbrace{[3Fe\text{-}4S]^{-1} \longleftrightarrow [3Fe\text{-}4S]^{2-}}_{\text{Couple C}'} \qquad (1)$$

The manner in which reduction potentials vary with pH indicates the *net* uptake of H^+ that accompanies electron transfer. Firstly, however, we need to consider

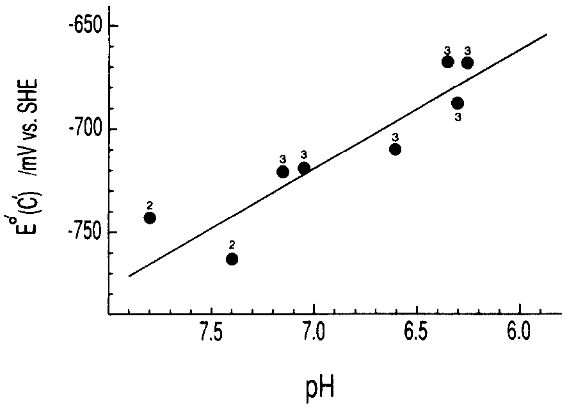

Fig.4. Variation of $E^{o\prime}$ for waves C′ with electrolyte pH. Black circles show average of values obtained from the number of determinations included (indicated). The least-squares fit to a simple linear relationship is shown. The slope is −57 mV, with $E^{o\prime}$ = −720 mV at pH 7.0.

the complex broadening of reduction wave C′ that is apparent as the pH or scan rate is increased (fig.2). Such broadening must be associated with kinetic effects that separate the reduction process into consecutive one-electron steps. (Note that this can occur while the more concerted nature of the reoxidation reaction is retained.) One explanation is that further reduction of the one-electron intermediate depends upon a chemical step such as *rearrangement*. If this became rate-determining under conditions of high pH/high scan-rate, the waveform would take on a drawn-out 'tail' as is indeed apparent. Such complexity may provide the explanation as to why, for Fd III, couple C′ appears restricted largely to molecules whose lifetime at the electrode surface is extended through strong adsorption. With this to consider, interpretation of $E^{o\prime}$ values for couple C′ becomes difficult. Indeed measurement itself is subject to error, due to broadening of the reduction wave at high pH. Data for the pH dependence of $E^{o\prime}$, shown in fig.4, should thus be treated with caution. Taking the slope $d(E^{o\prime})/d(pH)$ to be −57 mV, as obtained by a linear regression analysis, the implication is that two-electron reduction is accompanied by net binding of *two* H^+. However, curvature in this plot, reflecting p*K* values in the experimental pH range, cannot be ruled out until more data have been established.

In conclusion, we present evidence that $[3Fe\text{-}4S]^0$ clusters can undergo further reversible reduction with transfer of H^+. This occurs at a potential that is too low to be accessed through reduction by dithionite (lower useful limit at pH 7 is ca. −550 mV [10]) but is not significantly more negative than the $[4Fe\text{-}4S]^{2+/1+}$ couple of *Azotobacter chroococcum* Fd I ($E^{o\prime}$ = −645 mV [1]). For Fd III from *Desulfovibrio africanus* the one-electron reduced species is relatively unstable and a second electron is added readily to give a species that is sufficiently stable to survive for reoxidation after an incubation time of a minute or more. Some quite remarkable chemistry is thus implicated since the two-oxidation-level restriction observed for other protein-bound clusters is evidently relaxed. Binding of H^+ may occur directly at the cluster, either intact or rearranged, or at protein sites 'activated' by rearrangement. In terms of formal valencies it is most appropriate to assign to each Fe an oxidation state of II, but another possibility is that the equivalents are held as a coordinated hydrogen species, perhaps H^-. It remains to be seen whether the novel species involved, either in this protein or others, can be generated in bulk and isolated for spectroscopic characterisation and, significantly, whether this chemistry has any physiological importance.

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